DOI: 10.15255/KUI.2017.036
KUI-8/2018
Original scientific paper
Received August 31, 2017
Accepted October 20, 2017

Potential Use of Apple Polyphenol Oxidase for Bioremediation of Phenolic Contaminants

A. Šalić,[a] *I. Strelec*,[b] *B. Zelić*,[a] and *M. Tišma*[b*]

[a] University of Zagreb, Faculty of Chemical Engineering and Technology, Marulićev trg 19, HR-10 000 Zagreb, Croatia

[b] Josip Juraj Strossmayer University of Osijek, Faculty of Food Technology Osijek, Franje Kuhača 20, HR-31 000 Osijek, Croatia

Abstract

Phenolic compounds, such as catechol, are released into the environment from a variety of industrial sources and they present a serious ecosystem burden. This work examined the possibility of using partially purified apple polyphenol oxidase (PPO) for bioremediation of phenolic contaminants. In order to optimize process conditions, the optimal pH and temperature for PPO activity were determined, while PPO affinity toward various phenols, as well as the effect of some salts and organic solvents which can be found in wastewaters, was used to confirm applicability of PPO in wastewater treatment. It was found that partially purified apple PPO shows maximal activity at pH 6.8 and 25 °C, but exhibits more than 85 % of its maximal activity in pH range from 5 to 8, and more than 90 % of activity in temperature range from 10 to 50 °C. PPO showed high affinity for various diphenols, but lack of affinity toward monophenols. Sodium tetraborate decahydrate moderately inhibited PPO activity, while exposure of PPO to the presence of organic solvents (φ = 5 %) caused 40 % loss in its activity. Catechol oxidation by PPO performed for just 5 min in a batch reactor at optimal process conditions resulted in 25 % conversion. Based on obtained data, it seems that partially purified apple PPO has reasonable potential in wastewater treatment.

Keywords

Catechol oxidation, enzymatic wastewater treatment, phenolic contaminants, polyphenol oxidase (PPO)

1 Introduction

Wastewater discharged from a number of processing industries, such as production of rubber, dyes, pesticides, colours, plastics, pharmaceuticals, and cosmetics, contains dissolved organic pollutants such as phenol and substituted phenolic compounds.[1] The United States Environmental Protection Agency (EPA) has ranked phenols the 11th on a list of 126 toxic chemicals that have been designated as priority pollutants.[2] Their concentration usually ranges from 100 to 1000 mg l^{-1} and they present a serious toxic and hazard threat to the environment.[3] According to some regulations, the concentration of phenols in the industrial effluents should not exceed 0.05 to 1 mg l^{-1} for safe discharge in sewage and in marine environment.[4] Many different methods, physical and chemical (destructive oxidation, adsorptive micellar flocculation, ultrafiltration and adsorption on organic and inorganic compounds) and biological (phytoremediation and microbiological removal), have been applied for removal of those toxic compounds from wastewaters.[4,5] Although chemical and physical methods have been investigated exhaustively, they have many drawbacks, such as high costs, incomplete removal, formation of hazardous by-products that are even more toxic than phenols, low efficiency, high energy requirements and/or applicability only in a low range of concentrations.[6] On the other hand, there is a growing recognition that enzymes can be used in many remediation processes to target specific pollutants for treatment.[7] Application to different substrates, operation at high and low contaminant concentrations over a wide pH, temperature and salinity range, and easy control are just some of the advantages of enzymatic processes.[8] The idea of using oxidoreductive enzymes, like polyphenol oxidase (PPO) and peroxidase, for wastewater treatment was developed in the early 1980s.[9] PPOs became interesting because they are widely distributed through different microorganisms, plants and animals, and specific to a wide range of phenolic substrates transforming them to less toxic and insoluble quinones[5], making the separation much easier. They can be applied as free or immobilized enzymes, purified or crude extract. Among them, the least desirable is the use of purified free enzyme because they are non-reusable and highly sensitive towards denaturation agents. Therefore, immobilized enzyme, partially purified and even crude extract of PPO are more desirable forms.[10,11] Researchers have found that the presence of different enzymes and isoenzymes along with other non-enzyme constituents in crude extract permits the extract to function under a wider range of conditions (pH, temperature, polyphenol concentration), and with more substrates than would be possible using single enzymes.[12]

This study reports the possibility of using partially purified PPO from apple variety Idared for catechol removal from aqueous media. Catechol was chosen since it provokes changes in the function of erythrocytes at doses as low as 50 μg l^{-1} *i.e.* that dose for phenol is 250 μg l^{-1}.[13] Characterization of partially purified PPO from apple variety Idared was studied in terms of pH, temperature, and storage stability. Besides catechol, several different phenolic compounds were studied as possible substrates for partially purified apple PPO. The focus was also on the degree of

* Corresponding author: Assoc. Prof. Marina Tišma, PhD
e-mail: mtisma@ptfos.hr

inhibition by different inhibitors, to help predict the behaviour of the apple enzyme in complex media, such as in wastewater loaded with different phenolic compounds.

2 Experimental

2.1 Chemicals

Ammonium sulphate, acetone, dimethyl sulfoxide (DMSO), ethanol, ethylenediaminetetraacetic acid disodium salt (EDTA-2Na), and sodium chloride were purchased from Kemika (Croatia). Bradford reagent was purchased from Bio-Rad (Germany). L-3,4-dihydroxyphenylalanine (L-DOPA), (+)-catechin, caffeic acid, chlorogenic acid, (-)-epicatechin, 4-methylcatechol, *trans*-ferullic acid, (-)-epigallocatechin-3-gallate, bovine serum albumin, sodium tetraborate decahydrate, and catechol were purchased from Sigma-Aldrich (USA). L-tyrosine, sodium phosphate monobasic monohydrate, and sodium phosphate dibasic dihydrate were purchased from Merck (Germany), while Triton X-100 was purchased from Fluka (Germany).

2.2 Partial purification of apple polyphenol oxidase

Apples (*Malus domestica* cv. *Idared*) of commercial maturity, marked as Croatian product, purchased at a local store (Konzum, Croatia) were used as starting material for isolation of PPO. Apples of the variety Idared were chosen since they are the most common cultivar in Croatia (60 % of total apple production) and 4th in the European Union.[14]

Polyphenol oxidase from apples was partially purified by ammonium sulphate (30–80 %), and subsequent ice-cold acetone (−20 °C) precipitation according to *Strelec et al.*[15] Brownish acetone precipitate collected by centrifugation (15.000 g, 20 min, 4 °C; Heraeus Multifuge 3L-R Centrifuge, Heraeus, Germany) containing PPO (5018±198 U mg^{-1}) was stored at −20 °C and used for all experiments.

2.3 Determination of protein concentration

Protein concentrations in extracts and PPO solutions were measured according to the method of *Bradford*[16] using bovine serum albumin as the standard.

2.4 PPO activity assay

PPO activity was determined spectrophotometrically at 25 °C using a double-beam spectrophotometer Specord 200 (AnalytikJena, Germany), by measuring the absorbance at 475 nm or 420 nm, depending on the substrate. Change in absorbance was recorded every 10 s during 100 s for all substrates, except L-tyrosine, where the change in absorbance was recorded every 30 s during 15 min. One unit of PPO activity (U) was defined as the change in absorbance of 0.001 *per* min and *per* ml of enzyme. All the measurements were performed in triplicate, and in the 95 % confidence interval the results showed no significant difference.

2.5 Determination of pH and temperature optimum

The influence of pH on PPO activity was measured at T = 25 °C in the pH range from 5 to 9 using various buffers: a) 0.1 mmol l^{-1} sodium acetate buffer of pH 5 and 6; b) 0.1 mmol l^{-1} sodium phosphate buffer pH 6, 6.8, 7 and 8, and c) 0.1 mmol l^{-1} Tris-HCl buffer of pH 8 and 9. L-DOPA (10 mmol l^{-1}) was used as a model substrate.

The influence of temperature on PPO activity was measured in temperature range between 10 and 50 °C, while thermal stability of the enzyme was determined by incubating the enzyme solution at temperature of 4 °C and 25 °C up to 160 h. In both cases, L-DOPA (10 mmol l^{-1} in 0.1 mmol l^{-1} phosphate buffer, pH 6.8) was used as a substrate.

All the measurements were performed in triplicate, and in the 95 % confidence interval the results showed no significant difference.

2.6 Determination of enzyme stability

The stability of PPO solution (250 U mg^{-1} of PPO suspended in 0.1 mmol l^{-1} phosphate buffer, pH 6.8, containing Triton X-100 (φ = 0.1 %)) was monitored during seven days of storage at 4 and 25 °C, while storage stability of partially purified PPO was monitored during 228 days of storage at −20 °C. All the measurements were performed in triplicate, and in the 95 % confidence interval the results showed no significant difference.

2.7 Substrate specificity of apple PPO

Substrate specificity of PPO was determined using nine different substrates: L-3,4-dihydroxyphenylalanine (L-DOPA), (+)-catechin, caffeic acid, chlorogenic acid, (-)-epicatechin, (-)-epigallocatechin-3-gallate, 4-methylcatechol, *trans*-ferullic acid and L-tyrosine. Reaction mixture for PPO activity determination contained 1, 2.5, or 10 mmol l^{-1} substrate solution in 0.1 mmol l^{-1} phosphate buffer, pH 6.8, and 250 U mg^{-1} of PPO suspended in 0.1 mmol l^{-1} phosphate buffer, pH 6.8 at T = 25 °C, containing Triton X-100 (φ = 0.1 %). Increase in absorbance at 420 nm ((+)-catechin, caffeic acid, chlorogenic acid, (-)-epicatechin, (-)-epigallocatechin-3-gallate, 4-methylcatechol, *trans*-ferullic acid) or 475 nm (L-DOPA and L-tyrosine) was measured. All the measurements were performed in triplicate, and in the 95 % confidence interval the results showed no significant difference.

2.8 Effect of inhibitors and organic solvents on PPO activity

PPO activity was measured in the presence of three different inhibitors (ethylenediaminetetraacetic acid disodium salt, sodium chloride, and sodium tetraborate decahydrate (borax)) at three different concentrations in the reaction mixture (0.1, 1 and 10 mmol l^{-1}) containing 10 mmol l^{-1} L-DOPA as a substrate (T = 25 °C).

The effect of two organic solvents, dimethyl sulfoxide and ethanol, on PPO activity was measured at three different solvent volume percentages (1, 2 and 5 %) in the reaction containing 10 mmol l^{-1} L-DOPA as a substrate (T = 25 °C).

All the measurements were performed in triplicate, and in the 95 % confidence interval the results showed no significant difference.

2.9 Measurements of catechol concentration

Catechol concentration was measured using HPLC (LC-AT10, Shimadzu, Japan) at 30 °C with a reverse phase C18 125 mm x 4 mm x 5 µm column (LiChrospher 5u RP-18 100 A, Phenomenex, USA), and a UV detector at 280 nm according to the method described by *Jurinjak Tušek et al*.[17]

2.10 Catechol oxidation

Catechol oxidation was performed in a glass batch reactor (V = 50 ml) with reaction volume of 10 ml. The reaction was carried out at 25 °C in phosphate buffer (0.1 mmol l^{-1}, pH 6.8). Initial concentration of catechol was 7.7 mmol l^{-1}. The reaction started with the addition of 0.1 ml of PPO suspension to 7.7 mmol l^{-1} aqueous catechol solution to give a final PPO concentration in the reactor of 0.0011 mg ml^{-1}. The reaction mixture was stirred with a magnetic stirrer (800 rpm, Tehtnica, Czech Republic) and catechol concentration in the samples was monitored by HPLC as described above. Experiments were performed without additional aeration. An oximeter (Optical Oxygen Meter – FireStingO_2, Denmark) was used for measurement of dissolved oxygen concentration in the reaction mixture.

2.11 Modelling and data handling

Enzyme operational stability decay rate was described by the first order kinetics according to Eq (1).

$$\frac{dS.A.}{dt} = -k_d \cdot S.A. \quad (1)$$

where *S.A.* is specific activity (U mg^{-1}), t is time (h), and k_d is decay rate constant (h^{-1}). Decay rate constant was estimated by the nonlinear regression analysis using the least squares method implemented in the SCIENTIST software (MicroMath®, Salt Lake City, USA). It was evaluated by fitting the model to the experimental data. The calculated data were compared with the experimental data, recalculated in the optimization routine, and fed again to the integration step until a minimal error between experimental and integrated values was achieved. The residual was defined as the sum of the squares of the differences between the experimental and the calculated data. "Episode" algorithm for the stiff system of differential equations, implemented in the SCIENTIST software, was used for simulations of batch reactor mathematical models.

3 Results and discussion

3.1 Effect of pH on PPO activity

Once the PPO was partially purified (*S.A.* = 5018±198 U mg^{-1}), the effect of pH in the range from 5 to 9 on PPO activity was determined. This range was chosen based on previous reports where different authors reported that optimal pH values range from 5 to 7.[18–21] It was found that optimal pH correlates with literature data (Fig. 1) but, also, that it depends on the chosen buffer. When the 0.1 mmol l^{-1} sodium acetate buffer was used, pH optimum was determined to be 6, while when 0.1 mmol l^{-1} sodium phosphate was used, the optimal pH was 6.8. Therefore, 0.1 mmol l^{-1} sodium phosphate was used in all further experiments. Additionally, *Ni Eidhin et al*.[19] reported that activity of PPO from Bramley's Seedling apple sharply decreases at pH higher than 7 (for pH 7.5, enzyme retained only 20 % of its initial activity). The same effect was not noticed in this research using partially purified apple PPO. It was found that, in the pH range from 5 to 8, PPO retains more than 85 % of its initial activity, indicating that it is more stable than the PPO from Bramley's Seedling apple.

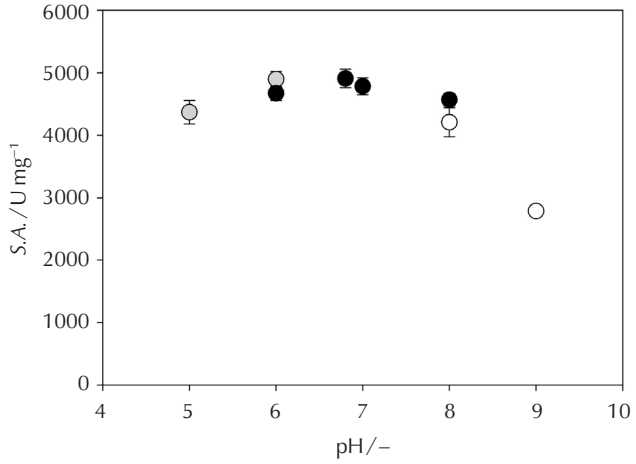

Fig. 1 – Effect of pH on PPO activity; ● 0.1 mmol l^{-1} sodium acetate buffer, ● 0.1 mmol l^{-1} sodium phosphate buffer and ○ 0.1 mmol l^{-1} Tris-HCl buffer

Slika 1 – Utjecaj pH-vrijednosti na aktivnost enzima PPO; ● 0,1 mmol l^{-1} natrijev acetatni pufer, ● 0,1 mmol l^{-1} natrijev fosfatni pufer i ○ 0,1 mmol l^{-1} Tris-HCl pufer

3.2 Effect of temperature on the PPO activity and stability

PPO activity was determined in the temperature range from 10 to 50 °C in 0.1 mmol l^{-1} phosphate buffer at pH 6.8. It was found that optimal temperature for partially purified PPO was 25 °C, but enzyme retained more than 90 % of its initial activity in temperature range from 10 to 50 °C (Fig. 2). Observed data are opposite to the report of *Ni Eidhin et al*.[19] who found that PPO from Bramley's Seedling apple shows optimal temperature at 30 °C, and retains from 60 to 80 % of its initial activity in temperature range from 10 °C to 50 °C.

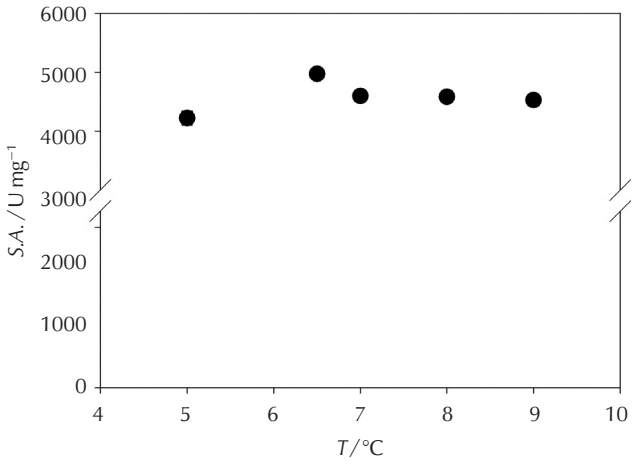

Fig. 2 – Effect of temperature on PPO activity
Slika 2 – Utjecaj temperature na aktivnost enzima PPO

PPO stability during storage was monitored once the PPO was partially purified and stored (−20 °C). Samples were taken during 228 days, and the results are presented in Fig. 3a. According to the obtained results, it can be concluded that no change in activity during 228 days had occurred.

In order to investigate PPO stability suspended in buffer (0.1 mmol l^{-1} phosphate buffer, pH 6.8, containing Triton X-100 (φ = 0.1 %)) and stored during 164 h at two different temperatures (4 and 25 °C), the samples were withdrawn with time and PPO activity was measured (Fig. 3b). As may be observed, the PPO in solution was still stable with a slight decrease in activity when stored at 4 °C. The temperature of 25 °C (shown to be optimal temperature for substrate oxidation) had a significant effect on PPO stability. It was noticed that PPO retained less than 50 % of its initial activity after 168 h.

In order to predict the time when the activity will decrease under 10 % of PPO initial activity, decay rate constants were determined assuming the first order kinetics. It was calculated that PPO stability decay rate constant for PPO stored at 4 °C was 0.00011±0.00002 h^{-1} and 0.00442±0.00017 h^{-1} for PPO stored at 25 °C. Based on obtained results and according to the mathematical model predictions, after seven months of storage at 4 °C, PPO will lose 50 % of its initial activity and 90 % after more than two years. On the other hand, when the activity was measured at 25 °C, 50 % of activity was lost after six days and 90 % after 21 days. Considering long-term processes conducted at 25 °C, obtained result is not so promising, but in order to overcome that disadvantage, biocatalyst immobilization is often implemented.[22] Immobilization of PPO can protect it from denaturation by organic co-solvents, increase their stability in general,[23] facilitate the separation of reaction products,[7] and maintain good catalytic efficiency over many reaction cycles.[24,25] Thus, this could be a possible solution for long-term processes.

3.3 Catechol oxidation catalysed with partially purified apple PPO

As mentioned in the introduction, catechol is released into the environment from a variety of industrial sources[1], and together with different polyphenolic compounds, presents a serious ecosystem burden.[26] Therefore, the removal of catechol from water or wastewaters is of great importance. Many reports have suggested that PPO has high affinity towards catechol,[18,19,27–30] therefore, catechol was chosen as a model substrate in wastewater purification. Batch mode catechol oxidation was performed in 10 ml scale. The reaction was carried out at 25 °C in phosphate buffer (0.1 mmol l^{-1}, pH 6.8), which was determined as optimal for PPO. Initial concentration of catechol was 7.7 mmol l^{-1}, while initial oxygen concentration was determined to be 0.186 mmol l^{-1}. The mixture was not aerated additionally, so the oxygen supply was provided only through the boundary layer atmosphere/reaction mixture. Results are presented in Fig. 4.

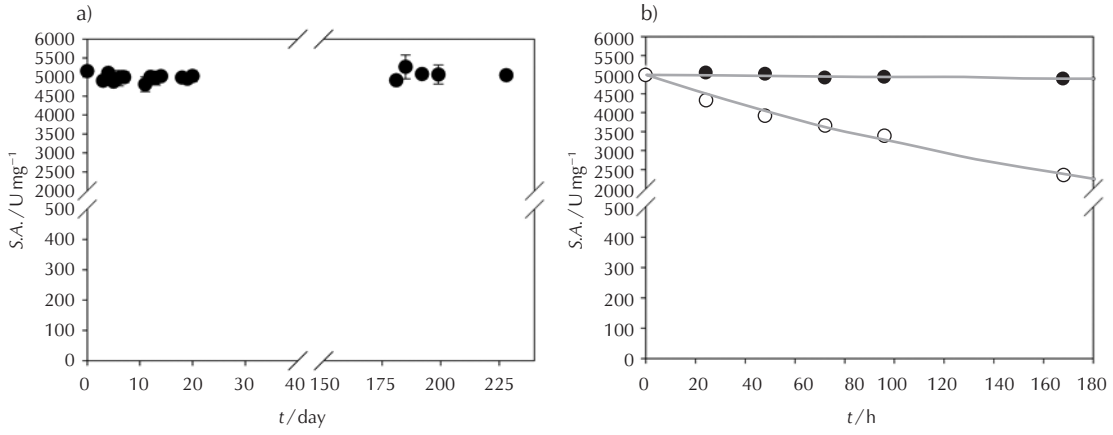

Fig. 3 – Temperature stability of PPO at a) −20 °C and b) ● 4 °C and ○ 25 °C, — mathematical model
Slika 3 – Temperaturna stabilnost enzima PPO pri a) −20 °C i b) ● 4 °C i ○ 25 °C, — matematički model

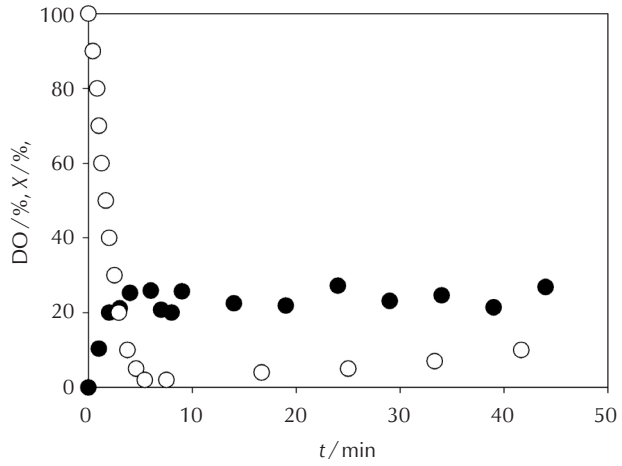

Fig. 4 – Catechol oxidation in a batch reactor (● catechol conversion, *X*; ○ dissolved oxygen concentration, DO)
Slika 4 – Oksidacija katehola u šaržnom reaktoru (● konverzija katehola, *X*; ○ koncentracija otopljenog kisika, DO)

Catechol conversion of 25 % was achieved after 5 min of reaction (Fig. 4). According to the results of dissolved oxygen concentration (Fig. 4), reaction time of 5 min was also the time when all the oxygen dissolved in the mixture was consumed, which confirmed that PPO is an oxygen-dependent enzyme, as well as that oxygen is the second substrate in the reaction. The obtained product of catechol oxidation is probably quinone,[31] which plants usually utilize to synthesize melanins – pigments that cause the damaged tissues to turn reddish-brown[32,33] and it is less toxic than catechol.

In the literature, for polyphenol removal, enzyme laccase is usually proposed (L-DOPA,[34] L-DOPA and catechol,[17] coniferyl alcohol[35]). Laccase is an enzyme that belongs to the group of effective enzymes capable of catalysing the oxidation of different phenolics. Research performed by *Tišma et al.*[34] confirmed that laccase can be used for L-DOPA oxidation and that the reaction is strongly dependent on dissolved oxygen concentration; therefore, continuous aeration had great effect on reaction rate. Similar results were obtained in the research performed by *Jurinjak* Tušek *et al.*[17] As for the results of catechol oxidation, the results obtained in this research (maximal conversion) were similar to the results obtained when laccase was used for catechol oxidation where the maximal conversion of 30 % was achieved (data not presented). The only significant difference was the reaction time; whereas 5 min was enough for PPO, it took almost 2 h to achieve the same conversion with enzyme laccase. Obtained results indicate that partially purified PPO could be a better solution than laccase for polyphenol oxidation/removal. Also, with additional process optimization (mainly continuous aeration), even better results (higher conversion) could be expected.

3.4 Substrate (polyphenol) specificity of partially purified apple PPO

The composition of wastewaters is never simple, and it contains a variety of organic and inorganic compounds. Among them, there are different phenolic compounds, not just catechol.[1] In order to determine which other polyphenolic compounds could be removed/oxidised by partially purified PPO, PPO specificity was investigated towards L-DOPA (-)-epicatechin, (-)-epigallocatechin-3-gallate, (+)-catechin, caffeic acid, chlorogenic acid, 4-methylcatechol, L-tyrosine and *trans*-ferullic acid.

The reaction was performed under optimal conditions regarding pH and temperature (0.1 mmol l^{-1} phosphate buffer, pH 6.8; T = 25 °C). Three different substrate concentrations (1, 2.5, and 10 mmol l^{-1}) were studied, and the results are presented in Table 1.

Table 1 – Substrate specificity of partially purified PPO from apple variety Idared
Tablica 1 – Supstratna specifičnost djelomično pročišćenog enzima PPO iz sorte jabuka Idared

Substrate Supstrati	c / mmol l^{-1}	Relative activity / %* Relativna aktivnost / %
L-DOPA	10.0	100.0
	2.5	28.8
	1	12.3
(-)-epicatechin (-)-epikatehin	10.0	272.8
	2.5	211.5
	1	26.9
(-)-epigallocatechin-3-galate (-)-epigalokatehin-3-galat	10.0	8.6
	2.5	5.3
	1	0.5
(+)-catechin (+)-katehin	10.0	407.4
	2.5	251.0
	1	28.3
caffeic acid kavena kiselina	10.0	62.5
	2.5	60.6
	1	11.9
chlorogenic acid klorogenska kiselina	10.0	116.0
	2.5	81.1
	1	12.8
4-methylcatechol 4-metilkatehol	10.0	255.8
	2.5	162.8
	1	9.3
L-tyrosine L-tirozin	10.0	0.0
	2.5	0.0
	1	0.0
trans-ferullic acid *trans*-ferulinska kiselina	10.0	0.0
	2.5	0.0
	1	0.0

*Relative activities are calculated as % of activity determined with L-DOPA (10 mmol l^{-1})

The highest activity was achieved using catechin, followed by epicatechin and 4-methylcatechol, then chlorogenic acid, L-DOPA, caffeic acid, and epigallocatechin-3-gallate.

Affinities towards mentioned substrates were expected, since they can all be found in apples.[19] It was found that PPO has no activity towards monophenols, L-tyrosine and *trans*-ferullic acid. Tyrosine has already been reported to be a poor substrate for apple PPO,[15,19,36] but it was shown to be a substrate for PPO isolated from other fruits, such as jackfruit.[28] In general, partially purified apple PPO had higher affinity towards di-phenolic then mono-phenolic compounds. Similar results were obtained with PPO originated from different fruits, like jackfruit,[28] Barbados cherry,[37] and other plant species, like peppermint,[38] dormant saffron[39] and butter lettuce,[40] indicating that they can also be used for polyphenol removal, as individual enzymes or in combination with others (for example, if the PPO is isolated from different fruit waste).

Briefly, partially purified PPO could be effectively used for the removal of the most studied polyphenols (*i.e.* diphenol), with the exception of monophenols, such as L-tyrosine and *trans*-ferullic acid. For monophenol removal, some other enzymes or PPO from different sources should be used.

3.5 Effect of inhibitors and organic solvents on PPO activity

Wastewaters can also contain many compounds that can inhibit or deactivate PPO. To determine the effect of different inhibitors on partially purified apple PPO activity, three different concentrations of inhibitors (0.1, 1, and 10 mmol l^{-1}) were investigated. The PPO activity without the presence of inhibitors was taken to be 100 %. As given in Table 2, sodium tetraborate-decahydrate showed 69 % of inhibition on partially purified PPO activity when used in the highest investigated concentration (10 mmol l^{-1}).

Table 2 – Effect of inhibitors on PPO activity
Tablica 2 – Utjecaj inhibitora na aktivnost enzima PPO

Inhibitor	c / mmol l^{-1}	Inhibition / %* Inhibicija / %
sodium tetraborate decahydrate natrijev tetraborat dekahidrat	10.0	69
	1.0	14
	0.1	10
sodium chloride natrijev klorid	10.0	16
	1.0	10
	0.1	5
EDTA-2Na	10.0	17
	1.0	7
	01	6

* Inhibitions are expressed as % of inhibition compared with the activity determined with L-DOPA (10 mmol l^{-1}) without addition of inhibitor

The minimum inhibition of PPO activity was noticed when EDTA-2Na and sodium chloride were used, which is in agreement with reports of *Geetha et al.*[28], but opposite to the findings of *Ni Eidhin et al.*[19] Obtained results indicate that PPO could not be effectively used for polyphenol removal if the wastewater contains large amounts of sodium tetraborate decahydrate.

Data on the influence of organic solvents on partially purified PPO are outlined in Table 3. PPO activity decreased with the increase in the amount of investigated organic solvent, and enzyme activity at the highest concentration of solvent was inhibited by 39 or 40 %. Observed decrease in PPO activity in the presence of organic solvents could be attributed to enzyme denaturation.

Table 3 – Effect of organic solvent on PPO activity
Tablica 3 – Utjecaj organskih otapala na aktivnost enzima PPO

Organic solvent Organsko otapalo	φ / %	Inhibition / %* Inhibicija / %
ethanol etanol	5.0	40
	2.0	21
	1.0	11
DMSO	5.0	39
	2.0	22
	1.0	14

* Inhibitions are expressed as % of inhibition compared with the activity determined with L-DOPA (10 mmol l^{-1}) without addition of organic solvent

4 Conclusion

The study of catechol oxidation catalysed by partially purified apple PPO revealed the highest enzyme activity at pH 6.8, in phosphate buffer 0.1 mmol l^{-1} and at temperature of 25 °C. At optimized process conditions, partially purified PPO could be efficiently used for catechol removal from the model wastewater. Besides catechol, partially purified PPO also shows affinity towards several different diphenols, like catechin, epicatechin, 4-methylcatechol, chlorogenic acid, L-DOPA, caffeic acid, and epigallocatechin-3-gallate, but it shows no affinity towards the mono-phenolic substrates, such as L-tyrosine and *trans*-ferullic acid. In addition, the presence of sodium tetraborate-decahydrate moderately inhibits PPO activity; therefore, partially purified PPO cannot be used for the treatment of wastewaters where this compound is present in higher concentrations.

Based on the obtained results, it can be concluded that partially purified PPO has great potential in wastewater treatment. With some additional process optimisation (complex model solutions, additional aeration, enzyme immobilization, *etc.*), even better results could be expected. As a final step, apple waste (apple peel and especially the apple core contain the highest amount of PPO) should be studied in the process of polyphenol removal by using one waste stream (apple residue) in treatment of the other (wastewater loaded by phenols).

List of abbreviations and symbols
Popis kratica i simbola

DMSO – dimethyl sulfoxide
– dimetil sulfoksid

DO – dissolved oxygen concentration
– koncentracija otopljenog kisika

EDTA-2Na – ethylenediaminetetraacetic acid disodium salt
– dinatrijeva sol etilendiaminotetraoctene kiseline

L-DOPA – L-3,4-dihydroxyphenylalanine
– L-3,4-dihidroksifenilalanin

PPO – polyphenol oxidase
– enzim polifenol oksidaza

S.A. – specific activity
– specifična aktivnost

References
Literatura

1. *H. Chen, J. Yao, F. Wang, Y. Zhou, K. Chen, R. Zhuang, M. M. F. Choi, G. Zaray*, Toxicity of three phenolic compounds and their mixtures on the gram-positive bacteria *Bacillus subtilis* in the aquatic environment, Sci. Total Environ. **408** (2010) 1043–1049, doi: https://doi.org/10.1016/j.scitotenv.2009.11.051.
2. Environmental Protection Agency, Sampling and Analysis Procedure for Screening of Priority Pollutants, Environment Monitoring and Support Laboratory (1977) Cununnati, Ohio, USA.
3. Faust SD, Aly OM (1983) Chemistry of water treatment. Butterworth, Woburn.
4. *K. Shakir, H. F. Ghoneimy, A. F. Elkafrawy, Sh. G. Beheir, M. Refaat*, Removal of catechol from aqueous solutions by adsorption onto organophilic-bentonite, J. Hazard. Mater. **150** (2008) 765–773, doi: https://doi.org/10.1016/j.jhazmat.2007.05.037.
5. *S. Mukherjee, B. Basa, B. Bhunia, A. Dey, B. Mondal*, Potential use of polyphenol oxidases (PPO) in the bioremediation of phenolic contaminants containing industrial wastewater, Rev. Environ. Sci. Biotechnol. **12** (2013) 61–73, doi: https://doi.org/10.1007/s11157-012-9302-y.
6. *T. P. Chung, H. Y. Tseng, R. S. Juang*, Mass transfer effect and intermediate detection for phenol degradation in immobilized *Pseudomonas putida* systems, Process Biochem. **38** (2003) 1497–1507, doi: https://doi.org/10.1016/S0032-9592(03)00038-4.
7. *N. Duran, E. Esposito*, Potential applications of oxidative enzymes and phenoloxidase-like compounds in wastewater and soil treatment: a review, Appl. Catal. B-Environ. **28** (2000) 83–99, doi: https://doi.org/10.1016/S0926-3373(00)00168-5.
8. *J. Karam, J. A. Nicell*, Potential applications of enzymes in waste treatment, J. Chem. Technol. Biotechnol. **69** (1997) 141–153, doi: https://doi.org/10.1002/(SICI)1097-4660(199706)69:2<141::AID-JCTB694>3.0.CO;2-U.
9. *A. M. Klibanov, B. N. Alberti, E. D. Morris, L. M. Felshin*, Enzymatic removal of toxic phenols and anilines from waste waters, J. Appl. Biochem. **2** (1980) 414–421.
10. *M. Y. Arica*, Immobilization of polyphenol oxidase on carboxymethylcellulose hydrogel beads: preparation and characterization, Polym. Int. **49** (2000) 775–781, doi: https://doi.org/10.1002/1097-0126(200007)49:7<775::AID-PI454>3.0.CO;2-O.
11. *K. N. Niladevi, P. Prema*, Immobilization of laccase from *Streptomyces psammoticus* and its application in phenol removal using packed bed reactor, World J. Microbiol. Biotechnol. **24** (2008) 1215–1222, doi: https://doi.org/10.1007/s11274-007-9598-x.
12. *K. Yamada, T. Inoue, Y. Akiba, A. Kashiwada, K. Matsuda, M. Hirata*, Removal of *p*-alkylphenols from aqueous solutions by combined use of mushroom tyrosinase and chitosan beads, Biosci. Biotechnol. Biochem. **70** (2006) 2467–2475, doi: https://doi.org/10.1002/app.30947.
13. *B. Bukowska, S. Kowalaska*, Phenol and catechol induce prehemalytic and hemolytic changes in human erythrocytes, Toxicol. Lett. **152** (2004) 73–84, doi: https://doi.org/10.1016/j.toxlet.2004.03.025.
14. *F. Ivković*, The assortment of apples in production in the EU and Croatia and which varieties to plant, Glasnik zaštite bilja **5** (2011) 17–19. (Published in Croatian)
15. *I. Strelec, P. Burić, I. Janković, T. Kovač, M. Molnar*, Inhibitory effect of coumarin derivatives on apple (cv. Idared) polyphenol oxidase, Croat. J. Food Sci. Technol. **9** (2017) 57–65, doi: https://doi.org/10.17508/CJFST.2017.9.1.08.
16. *M. M. Bradford*, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. **72** (1976) 248–254, doi: https://doi.org/10.1016/0003-2697(76)90527-3.
17. *A. Jurinjak Tušek, M. Tišma, V. Bregović, A. Pričar, Ž. Kurtanjek, B. Zelić*, Enhancement of phenolic compounds oxidation using laccase from *Trametes versicolor* in a microreactor, Biotechnol. Bioprocess. E. **18** (2013) 686–696, doi: https://doi.org/10.1007/s12257-012-0688-8.
18. *M. Oktay, I. Küfreviolu, I. Kocaçalişkan, H.* Şakirolu, Polyphenoloxidase from Amasya apple, J. Food Sci. **60** (1995) 494–496, doi: https://doi.org/10.1111/j.1365-2621.1995.tb09810.x.
19. *D. M. Ni Eidhin, E. Murohy, D. O'Beirne*, Polyphenol oxidase from apple (*Malus domestica* Borkh. cv Bramley's Seedling): Purification strategies and characterization, J. Food Sci. **71** (2006) 51–58, doi: https://doi.org/10.1111/j.1365-2621.2006.tb12388.x.
20. *A. M. C. N. Rocha, M. P. Cano, M. A. M. Galeazzi, A. M. M. B. Morais*, Characterization of 'Starking' apple polyphenoloxidase, J. Sci. Food Agric. **77** (1998) 527–534, doi: https://doi.org/10.1002/(SICI)1097-0010(199808)77:4<527::AID-JSFA76>3.0.CO;2-E.
21. *C. A. Weemaes, L. R. Ludikhuyze, I. Van den Broeck, M. E. Hendrickx, P. P. Tobback,* Activity, electrophoretic characteristics and heat in activation of polyphenoloxidases from apples, avocados, grapes, pears and plums, Lebensm.-Wiss. Technol. **31** (1998) 44–49, doi: https://doi.org/10.1006/fstl.1997.0302.
22. *G. Stojkovič, I. Plazl, P.* Žnidaršič *Plazl*, L-Malic acid production within a microreactor with surface immobilised fumarase, Microfluid. Nanofluid. **10** (2011) 627–635, doi: https://doi.org/10.1007/s10404-010-0696-y.
23. *A. D'Annibale, S. R. Stazi, V. Vinciguerra, G. Giovannozzi Sermanni*, Oxirane immobilized *Lentinula edodes* laccase: stability and phenolics removal efficiency in olive mill wastewater, J. Biotechnol. **77** (2000) 265–273, doi: https://doi.org/10.1016/S0168-1656(99)00224-2.
24. *P. Brandi, A. D'Annibale, C. Galli, P. Gentili, A. S. N. Pontes*, In search for practical advantages from the immobilisation of an enzyme: the case of laccase, J. Mol. Catal. B-Enzym. **41** (2006) 61–69, doi: https://doi.org/10.1016/j.molcatb.2006.04.012.
25. *G. Palmieri, P. Giardina, G. Sannia*, Laccase-mediated Rema-

zol brilliant blue R decolorization in a fixed-bed bioreactor, Biotechnol. Progr. **2** (2005) 1436–1441, doi: https://doi.org/10.1021/bp050140i.

26. *J. Michałowicz, W. Duda*, Phenols – Sources and toxicity, Poli. J. Environ. Stud. **16** (2007) 347–362.
27. *J. R. Das, S. G. Bhat, L. R. Gowda*, Purification and characterization of a polyphenol oxidase from Kew cultivar of Indian pineapple fruit, J. Agric. Food Chem. **45** (1997) 2031–2035, doi: https://doi.org/10.1021/jf9607674.
28. *R. Y. Geetha, R. Arulvel, E. Selvarajan*, Studies of kinetic parameters and biochemical characteristics of polyphenol oxidase purified from jackfruit (*Artocarpus heterophyllus*) waste, Int. J. Pharm. Pharm. Sci. **6** (2014) 473–478.
29. *M. Siddiq, N. K. Sinha, J. N. Cash*, Characterization of polyphenoloxidase from Stanley plums, J. Food Sci. **57** (1992) 1177–1179, doi: https://doi.org/10.1111/j.1365-2621.1992.tb11292.x.
30. *P. Zhou, N. L. Smith, C. Y. Lee*, Potential purification and some properties of Monroe apple peel polyphenol oxidase, J. Agric. Food Chem. **41** (1993) 532–536, doi: https://doi.org/10.1021/jf00028a004.
31. *C. Queiroz, M. L. M. Lopes, E. Fialho, V. L. Valente-Mesquita*, Polyphenol Oxidase: Characteristics and mechanisms of browning control, Food Rev. Int. **24** (2008) 361–375, doi: https://doi.org/10.1080/87559120802089332.
32. *J. C. Espín, P. A. García-Ruiz, J. Tudela, R. Varón, F. García-Cánovas*, Monophenolase and diphenolase reaction mechanisms of apple and pear polyphenol oxidases, J. Agric. Food Chem. **46** (1998) 2968–2975, doi: https://doi.org/10.1021/jf971045v.
33. *M. J. Peñalver, L. G. Fenoll, J. N. Rodríguez-López, P. A. García-Ruiz, F. García-Molina, R. Varón, F. García-Cánovas, J. Tudela*, Reaction mechanism to explain the high kinetic autoactivation of tyrosinase, J. Mol. Catal. B-Enzym. **33** (2005) 35–42, doi: https://doi.org/10.1016/j.molcatb.2005.02.002.
34. *M. Tišma, P.* Žnidaršič-*Plazl, I. Plazl, B. Zelić, Đ. Vasić-Rački*, Modelling of L-DOPA oxidation catalysed by laccase, Chem. Biochem. Eng. Q. **22** (2008) 307–313.
35. *M. Tišma, P.* Žnidaršič-*Plazl, I. Plazl, B. Zelić, Đ. Vasić-Rački*, Oxidation of coniferyl alcohol catalysed by laccase from *Trametes versicolor*, Acta Chim. Slov. **57** (2010) 110–117.
36. *A. M. C. N. Rocha, A. M. M. B. Morais*, Characterization of polyphenoloxidase (PPO) extracted from *'Jonagored' apple*, Food Control **12** (2001) 85–90, doi: https://doi.org/10.1002/(SICI)1097-0010(199808)77:4<527::AID-JSFA76>3.0.CO;2-E.
37. *V. B. Anil Kumar, T. C. Kishor Mohan, K. Murugan*, Purification and kinetic characterization of polyphenol oxidase from Barbados cherry [*Malpighia glabra* L.], Food Chem. **110** (2008) 328–333, doi: https://doi.org/10.1016/j.foodchem.2008.02.006.
38. *D. Kavrayan, T. Aydemir*, Partial purification and characterisation of polyphenoloxidase from peppermint [*Mentha piperita*], Food Chem. **74** (2001) 147–54, doi: https://doi.org/10.1016/S0308-8146(01)00106-6.
39. *S. Saiedian, E. Keyhani, J. Keyhani*, Polyphenol oxidase activity in dormant saffron [*Crocus sativus* L.] corm, Acta Physiol. Plant. **29** (2007) 463–471, doi: https://doi.org/10.1007/s11738-007-0056-z.
40. *U. Gawlik-Dziki, Z. Złotek, M. Swieca*, Characterisation of polyphenol oxidase from butter lettuce [*Lactuca sativa var-capitata* L.], Food Chem. **107** (2008) 129–135, doi: https://doi.org/10.1016/j.foodchem.2007.07.068.

SAŽETAK

Potencijalna primjena polifenol oksidaze porijeklom iz jabuka u bioremedijaciji fenolnih onečišćujućih tvari

Anita Šalić,[a] *Ivica Strelec*,[b] *Bruno Zelić* [a] i *Marina Tišma*[b*]

Fenolni spojevi, poput primjerice katehola, dospijevaju u okoliš iz različitih industrija i predstavljaju ozbiljni ekološki problem. U ovom radu istraživana je mogućnost primjene djelomično pročišćene polifenol oksidaze (PPO) iz jabuke u bioremedijaciji različitih fenolnih onečišćujućih tvari. S ciljem optimiranja procesnih uvjeta, određeni su optimalni pH i temperatura za aktivnost PPO-a. Za provjeru mogućnosti primjene PPO-a u obradi otpadnih voda, mjeren je afinitet PPO-a prema različitim fenolnim spojevima kao i utjecaj nekih soli i organskih otapala na njenu aktivnost.

Djelomično pročišćen PPO ima maksimalnu aktivnost pri pH 6,8 i 25 25 °C, a više od 85 % maksimalne aktivnosti pokazuje u području pH od 5 do 8, a više od 90 % svoje maksimalne aktivnosti u temperaturnom rasponu od 10 to 50 °C.

Pokazano je da PPO ima veći afinitet prema različitim difenolnom spojevima, dok ne pokazuje afinitet prema monofenolnim spojevima. Natrijev tetraborat dekahidrat ima mali inhibitorni učinak na aktivnost PPO-a, dok utjecaj organskih otapala (φ = 5 %) uzrokuje 40 %-tni gubitak aktivnosti PPO-a. Oksidacijom katehola pomoću PPO-a pri optimalnim procesnim uvjetima u kotlastom reaktoru tijekom samo 5 minuta postignuta je 25 %-tna konverzija. Na temelju provedenih rezultata moguće je zaključiti da djelomično pročišćen PPO ima odgovarajući potencijal za primjenu u obradi otpadnih voda.

Ključne riječi

Oksidacija katehola, enzimska obrada otpadnih voda, fenolne onečišćujuće tvari, polifenol oksidaza (PPO)

[a] *Sveučilište u Zagrebu, Fakultet kemijskog inženjerstva i tehnologije, Marulićev trg 19, 10000 Zagreb*

[b] *Sveučilište Josipa Jurja Strossmayera u Osijeku, Prehrambeno-tehnološki fakultet Osijek, Franje Kuhača 20, 31000 Osijek*

Izvorni znanstveni rad

Prispjelo 31. kolovoza 2017.

Prihvaćeno 20. listopada 2017.